#16 1/7/03

Attorney's Docket No.: 07252-008001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Zyskind, J. W., et al.
Serial No. : 08/971,090
Filed : November 14, 1997
Title : METHOD FOR IDENTIFYING MICROBIAL PROLIFERATION GENES

Art Unit : 1655
Examiner : Jeffrey Fredman, Ph.D.

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.131

Sir:

1. I, R. Allyn Forsyth, am a co-inventor with Judith W. Zyskind, on the above-identified patent application, which was filed on November 14, 1997.

2. Judith W. Zyskind and I conceived of the claimed invention and reduced it to practice in the United States of America prior to February 14, 1997, which is the filing date of U.S. patent application serial no. (USSSN) 08/800,664, which is the priority document for U.S. Patent No. 5,955,275, filed March 4, 1997, and issued on September 21, 1999.

3. As evidence that Judith W. Zyskind and I conceived of the claimed invention and reduced the claimed invention to practice prior to February 14, 1997, I have enclosed as Exhibits A through C, photocopies of laboratory notebook pages, the dates of which have been redacted, but which are prior to February 14, 1997:

(a) Exhibit A contains a copy of one notebook page summarizing the results of experiments practicing a method of the invention that identifies microbial proliferation genes by introducing exogenous nucleic acids into a microorganism, the exogenous nucleic acid having substantial sequence identity to an endogenous microbial gene wherein the exogenous nucleic

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

Date of Deposit

Signature

Typed or Printed Name of Person Signing Certificate

acid is a random fragment or a random sequence; and identifying the endogenous gene as a microbial proliferation gene by comparing the proliferation or viability of the microorganism when the exogenous nucleic acid is expressed in or introduced into the microorganism with the proliferation or viability of the microorganism when the exogenous nucleic acid is not present or not expressed. Specifically, plasmid constructs containing random fragments of a bacterial genome (an *E. coli* genome) as inserts under the control of an inducible promoter were constructed. The inducible promoter is induced by "IPTG," a chemical that induces expression of the insert under the control of the promoter. The technique wherein random fragments are generated, cloned and transfected is designated as a "shotgun cloning strategy." These plasmids were moved into *E. coli*. The ability of the transfected bacteria to proliferate before and after induction of the inducible promoter was tested. A plasmid was identified that, when expressed in bacteria, inhibited growth of the microorganism after application of IPTG to induce expression of the gene fragment inserts. This plasmid, designated pJB3, was isolated, and the expressed insert was sequenced. This indicated that the insert comprised a fragment of an *E. coli* gene, designated secA, and that the insert had been expressed in the sense orientation. The gene secA is an essential growth gene in *E. coli*.

(b) Exhibit B contains copies of the notebook pages expressly referred to in the laboratory notebook page contained in Exhibit A: page 64B and pages 71B to 73B.

(c) Exhibit C contains copies of notebook pages numbered 93 to 95, describing experiments similar to those described above in paragraph (a). Page 94 illustrates a summary of this set of experiments. As above, bacterial growth and viability before and after induction of the inducible promoter were tested Three plasmids were found to inhibit growth and decrease the viability of the bacteria upon induction/ activation of their gene fragment inserts. As above, these plasmids were isolated, and the inserts of these plasmids were sequenced. The plasmid designated pJB3, the same as discussed in paragraph (a), showed significant negative effects on growth and viability. The two inserts from the plasmids designated pJB37 and pJB57, when expressed, also showed significant negative effects on growth and viability. Analysis of the pJB37 insert indicated that it was a gene expressed in a sense orientation and that, specifically, it

was a 546 base pair *PstI/HindIII* fragment of *lepA* (see declaration by Dr. R. Allyn Forsyth submitted with response dated June 4, 1999, for the instant application). Analysis of the pJB57 insert indicated that it comprised at least two genes: one gene, a 216 base pair *BamHI* fragment of *tsf* and *rspB* expressed in a sense orientation; and, a second gene, a 176 *EcoRI/ BamHI* fragment of *ddlB* expressed in an antisense orientation (see declaration by Dr. R. Allyn Forsyth, dated May 3, 1999). These genes (*lepA, tsf* and *rspB,* and *ddlB),* identified by the methods of the claimed invention, are essential for bacterial growth and viability.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted

Date: 10/26/2000

R Allyn Forsyth

R. Allyn Forsyth

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(a) Exhibit A contains a copy of one notebook page summarizing the results of experiments practicing a method of the invention that identifies microbial proliferation genes by introducing exogenous nucleic acids into a microorganism, the exogenous nucleic acid having substantial sequence identity to an endogenous microbial gene wherein the exogenous nucleic acid is a random fragment or a random sequence; and identifying the endogenous gene as a microbial proliferation gene by comparing the proliferation or viability of the microorganism when the exogenous nucleic acid is expressed in or introduced into the microorganism with the proliferation or viability of the microorganism when the exogenous nucleic acid is not present or not expressed. Specifically, plasmid constructs containing random fragments of a bacterial genome (an *E. coli* genome) as inserts under the control of an inducible promoter were

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These genes (*lepA, tsf* and *rspB,* and *ddlB),* identified by the methods of the claimed invention, are essential for bacterial growth and viability.

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Respectfully submitted

Date: August 4, 2000

Judith W. Zyskind

Judith W. Zyskind

10041853.doc

APPENDIX A
TO
DECLARATION OF JUDITH W. ZYSKIND

10047539.doc

Produced: or [redacted] shotgun cloning strategy

Size: 4.6 Kb

Insert: ≈ 850 bp (vec = 4.1~4.2) ~~BamHI~~ & PstI

Sensitivity to IPTG:

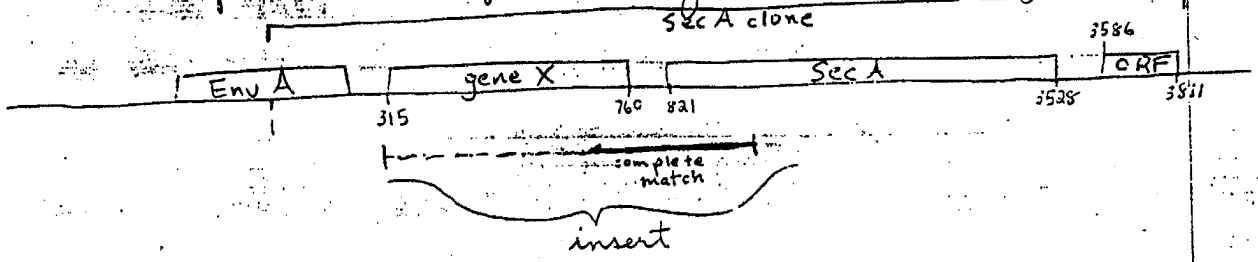
- on [redacted] IPTG growth experiment conducted (pg 64 B lab notebook) & pJB3 showed sensitivity
- other viable cell count experiments pgs 71 B – 73 B shows sensitivity as well (9.2 x 10^8 viable; control = 9.8 x 10^8)

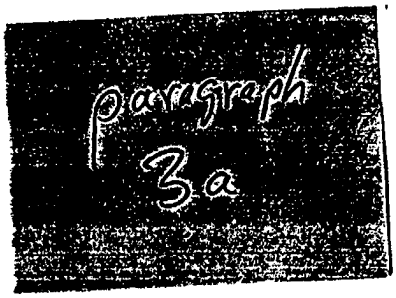
Sensitivity on Replica plate: no growth; revertant colonies day #2

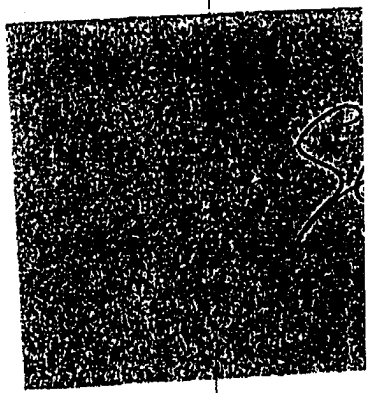
* pJB3 showed exact size of clones 2 & 7; even when cut w/ a third Restriction Enzyme Sau3AI.

Insert components: NCBI data bases / genbank

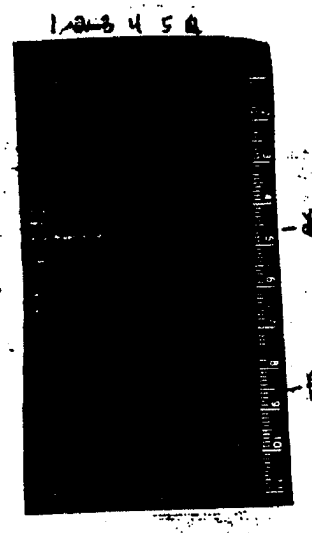
- matched up with a portion of the Sec A gene







Here I [redacted] 2 4 mutants to [redacted] nine by RE digest because of their initial size (about 4.8 – 5.5 Kb



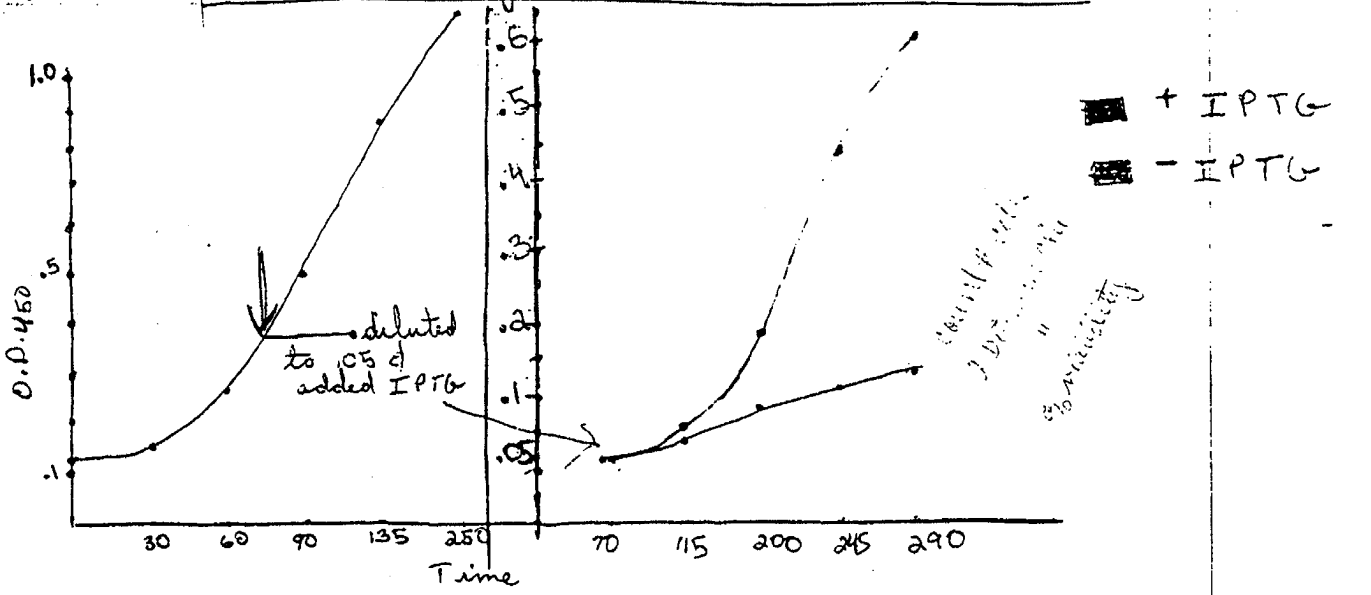
1hr 45 min @ 75 mA's 3% TEA agarose stained 40 min ft O.N.

1) 1 Kb ladder 2 λ
2) pJBI9 cut w/ HindIII & PstI 10 λ
3) " 12 " " " " } 100
4) " 13 " " " "
5) " 16 " " " "

Results: All had complete cutting but there doesn't seem to be any inserts released that are 1 Kb. The smalle [redacted] inserts are lanes 3 (900 bp) & 4 (900 bp).

Paragraph 3b

Evidence of IPTG sensitive cells



1-15x cells w/ pJBI3 were grown in LB at 37°C w/ Ap 100. Here, graphs show growth + & - IPTG. [redacted] the cells were induced w/ IPTG after [redacted] 70 min of growth to OD_{450} of 0.5, then grown for another 220 min. The slow growth of IPTG+ cells helps our decision to get pJBI3 sequenc

sequencing clone 3

- grew pJBI3, ON in 5 ml LB + Ap^{100}
- diluted back 1/200 (100 mls LB + Ap^{100})
- @ OD_{450} = .05 – .1 moved 50 mls to sidearm flask w/o IPTG
  " " " to sidearm flask w/ 100µM IPTG
- every 30' remove 1 ml of each culture; dilute out to 1×10^{-8} and plate on Ap^{100} + 100µM IPTG & Ap^{100} only

	−IPTG	+IPTG	
(1:10) T_0	~~.028~~ .07	.06	
T_{30}	.144	.13	
T_{60}	.27	.21	
T_{90}	.44	.28	
T_{120}	.60	.36	*
T_{150}	.85	.44	*
T_{180}	.97	.46	
T_{210}	.75(2)	.5	no plating
T_{240}	.85(2)	.58	
340	0.72(4)	.7	no plating
9:30 am T_{1360}	(47) 3.76 8	(43) 1.72 4	*

OD_{450}

Plating:

NI = noninduced
I = induced w/ 100µM IPTG

T_0	NI (10^{-5}, 10^{-6}, 10^{-7})	on +/− IPTG
	I (" , " , ")	on +/− IPTG
$T_{30,60}$	NI (10^{-6}, 10^{-7}, 10^{-8})	on − IPTG
	I (" , " , ")	on +/− IPTG
T_{90}	NI (" , " , ")	" " "
	I (" , " , ")	" " "
$T_{120,160,180}$	NI (" , " , ")	on − IPTG
	I (" , " , ")	on +/− IPTG
T_{210}	NI (" , " , ")	" " "
	I (" , " , ")	" " "

* 10^{-7} dilution was incorrect 100 λ culture / 990 ≅ 10^{-1} final dilutions are 10^{-7}, 10^{-8}, 10^{-9}

* collect the following for westerns

+IPTG culture

(3.84 mls)(1.72 OD) = 6.6 OD mls

$\frac{6.6\ OD}{V_T} = 50\ O.D.$ $V_T = 132$ µl

66 µl Solubilization Buffer + 66 µl 2X Loading

−IPTG culture

(1.92 mls)(3.76 OD) = 7.22 OD mls

$\frac{7.22}{V_T} = 50\ O.D.$ $V_T = 144$ µl

72 µl Sol. buffer + 72 µl 2X loading buffer

Viable cell count

10
B

Materials:

- 10 sidearm flasks w/ 100 mL L.B. + Ap^{100}
- 70 Ap^{100} plates
  - T_0 = 40 plates
  - T_{240} = 30 plates
- OD_{450} spec.
- IPTG @ 100 mM (add 100 µl / 100 ml LB for conc. of 100 µM)
- eppies with serial dil. set up: $T_0 \rightarrow 10^{-4}, 10^{-5}$; $T_{240} \rightarrow 10^{-3}, 10^{-4}, 10^{-5}$
- turn table

Procedure:

1) dilute all sidearm flasks 1:500 with O.N.'s 1-10 (200 µl ON → 100 mL LB amp)
2) agitate all cultures @ 37°C until OD_{450} has reached 0.1 – 0.2 on spec.
3) aliquot desired volumes from all cultures for serial dil. & plating at T_0
   ex) 10 µl culture → 990 H_2O = 10^{-2}
   10^{-2} → plate LB+amp = 10^{-3}
4) after aliquoting, immed. add IPTG to fin. conc. of 100 µM in all cultures, return to 37°C shaking, and resume with ser. dil. & plating at T_0
5) after 4 hr of 37°C shaking at T_{240}, aliquot desired volume of cultures and do as in step #3
6) put plates in incubator at 37°C
   * im planning to grow for 15 hr
   Too long / short

Training course

*wanted to check sensitivity of all clones created up to this point in order to see which were worth sequencing

- grew 4 ml O.N.'s of all clones: (in LB + Ap^{100} @ 3:
  1 – 28 clones
  29 – 42 subclones > 42 in all
- next day added 100 μM IPTG & grew for 30 hrs
-

data → over

Isolated NotI vector fragment (Sans origin) of pLEX 1B, 2B, 3B
There wasn't as much as expected.
Used Ultra clean for isolation.
Then cut 25 ul (vs 7 ul)

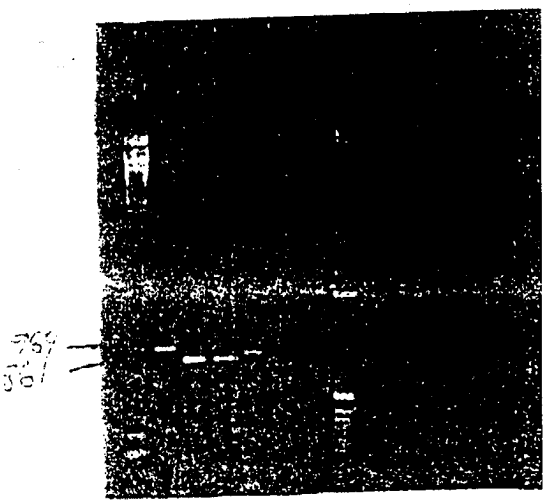
25.2 ul	pLEX 1B, 2B, 3B
~~5~~ 3.0	10X NEB #3
0.3	100X BSA
1.5	NotI (10 u/ul)
30.0	

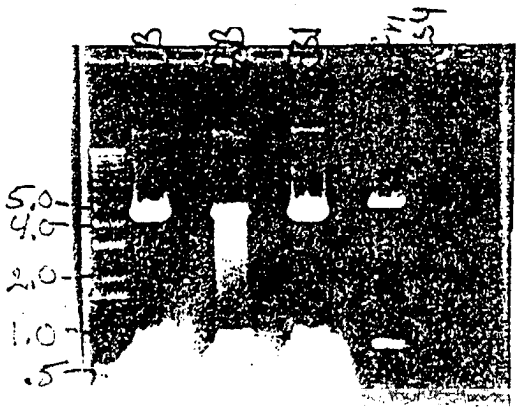
also cut [illegible]

[illegible]	[illegible]
10X NEB #3	5.0
100X BSA	0.5
H2O	[illegible]
	50.0

PCR [illegible]
PCR product = [illegible]
pJB 53, 55, 58
Were successfully [illegible]

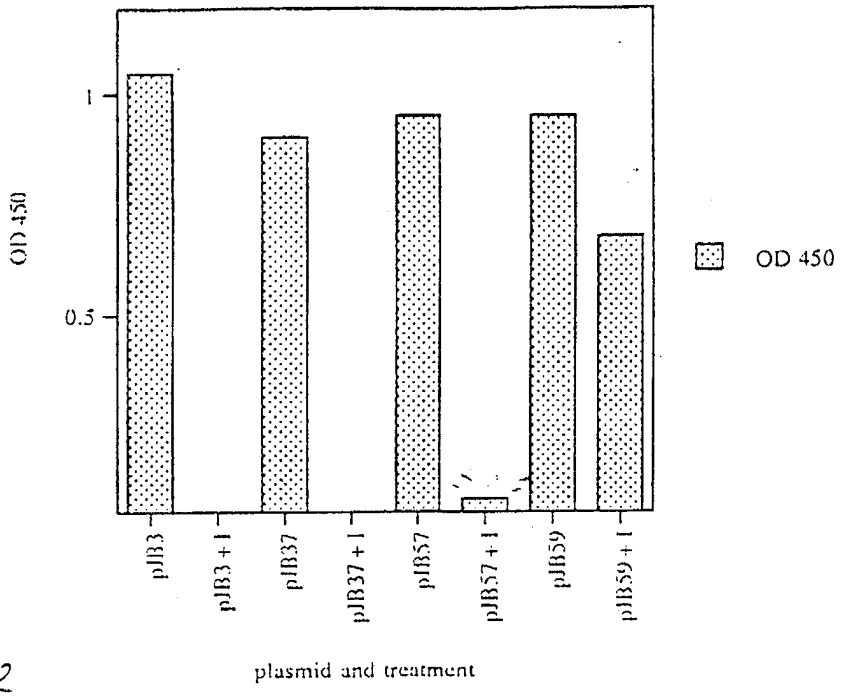
paragraph
3c





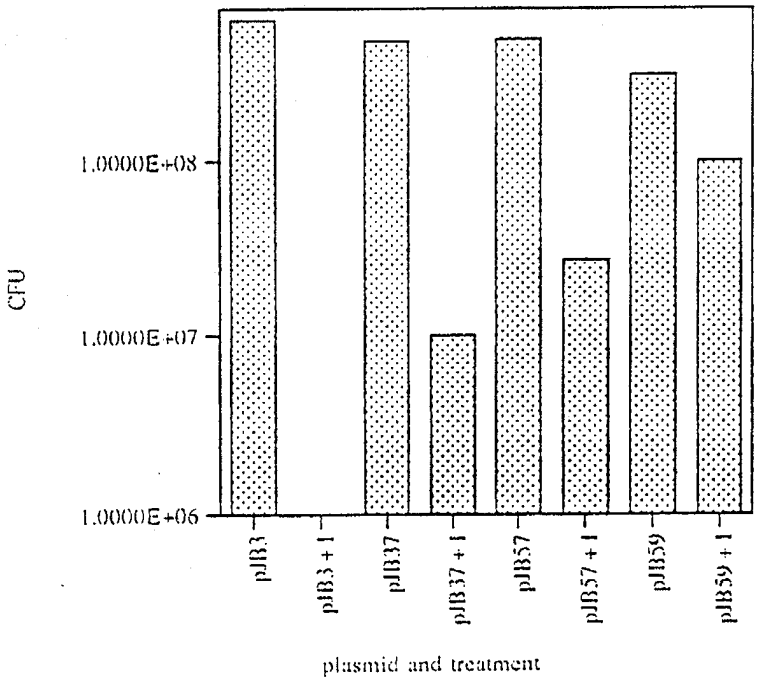
Used Ultra Clean kit to purify vector fragments of pLEX 1-3BS & pRTF11 away from Origin NotI fragments.
Resuspended silica pellet in 10 ul of H_2O used to resuspend pellets shown in 6/3/98 gel.

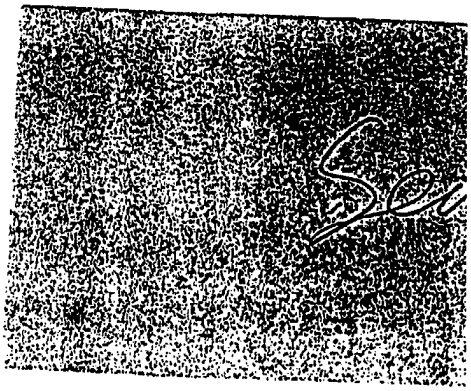
Growth of cells with Induction of Antisense RNA



JB3 secA sense
37 ~~dapB~~ asense
57 ddlB
59 ampG

Viability of cells carrying antisense plasmids with Induction





	1	2	3
	plasmid	OD 450	CFU
1	pJB3	1.05	6.0000E+08
2	pJB3 + I	0	1.0000E+06
3	pJB37	0.9	4.6000E+08
4	pJB37 + I	0.01	1.0000E+07
5	pJB57	0.95	4.7000E+08
6	pJB57 + I	0.08	2.7000E+07
7	pJB59	0.95	3.0000E+08
8	pJB59 + I	0.68	1.0000E+08
9			
10			

CONFIDENTIAL

Cultures were innoculated Sunday night (9^{30}pm) 1/10^{8} nto LBap^{100} ≠ 100um IPTG. 18 hours later at 3^{30}pm. OD_{450} was read and ~~cells~~ cultures were serially diluted and plated LBap^{100}.

PATENT
ATTORNEY DOCKET NO. 07252/008001

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Box AF
Assistant Commissioner for Patents
Washington, DC 20231

DECLARATION UNDER 37 C.F.R. §1.132 OF R. ALLYN FORSYTH

I, R. Allyn Forsyth, hereby declare as follows:

1. That I am a named inventor on U.S. patent application Serial No.: 08/971,090;

2. That I am presently employed by Elitra Pharmaceuticals, Inc., San Diego, California, as a Research Scientist and have been so employed since 1997;

3. That Elitra Pharmaceuticals is a licensee of the invention claimed in U.S. patent application Serial No.: 08/971,090;

4. That, subsequent to the filing date of U.S. patent application Serial No. 08/971,090, I or individuals under my supervision undertook a recharacterization of the plasmids listed in Table 1 of the application. The investigation revealed that the nature and orientation of the inserted sequences in certain plasmids is as indicated in the Table below.

Date of Deposit June 4, 1999
I hereby certify under 37 CFR 1.8(a) that this correspondence is being deposited with the United States Postal Service as **first class mail** with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Ann Pommier
(Ann Pommier)

Characteristics of Plasmids Listed in Table 1 of U.S. 08/971,090

Plasmid[1]	Insert	Orientation
pJB12	172 bp *E. coli* fragment, about 520 bp *E. coli* fragment plus about 700 bp fragment of *lepB*	172 bp: Unknown 520 bp: Unknown lep B: Antisense
pJB37	546 bp *PstI/HindIII* fragment of *lepA*	Sense
pJB40	546 bp *Pst/Hind III* fragment of *lepA*	Sense
pJB53	714 bp *PstI/HindIII* fragment of *viaA*	Sense
pJB57	216 bp *BamHI* fragment of *tsf* and *rpsB* plus 176 bp *EcoRI/BamHI* fragment of *ddlB*	*tsf/rpsB*: Sense *ddlB*: Antisense
pJB59	144 bp *BamHI* fragment of *ilvC* and 451 bp *EcoRI/BamHI* fragment of *orf1/ampG*	*ilvC*: Sense *orf1/ampG*:Antisense
pCW37	pJB37 insert in reverse orientation	Antisense
pCW53	pJB53 insert in reverse orientation	Antisense
pCW57	pJB57 inserts in reverse orientation	*tsf/rpsB*: Antisense *ddlB*: Sense
pCW59	pJB59 inserts in reverse orientation	*ilvC*: Antisense *orf1/ampG*:Sense
pAF37	Same insert as pJB37	Sense
pAF53	Same insert as pJB53	Sense
pAF57	Same insert as pJB57	*tsf/rpsB*: Sense *ddlB*: Antisense
pAF59	Same insert as pJB59	*ilvC*: Sense *orf1/ampG*:Antisense

[1] Inserts in plasmids designated pJB or pCW were cloned in the pLEX5BA vector; inserts in plasmids designated pAF were cloned in the pAFΩ vector.

5. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: May 3, 1999

R. Allyn Forsyth

R. Allyn Forsyth, Ph.D.

21679.M11